Maintenance of Intratumoral Androgens in Metastatic Prostate Cancer: A Mechanism for Castration-Resistant Tumor Growth

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Abstract

Therapy for advanced prostate cancer centers on suppressing systemic androgens and blocking activation of the androgen receptor (AR). Despite anorchid serum androgen levels, nearly all patients develop castration-resistant disease. We hypothesized that ongoing steroidogenesis within prostate tumors and the maintenance of intratumoral androgens may contribute to castration-resistant growth. Using mass spectrometry and quantitative reverse transcription–PCR, we evaluated androgen levels and transcripts encoding steroidogenic enzymes in benign prostate tissue, untreated primary prostate cancer, metastases from patients with castration-resistant prostate cancer, and xenografts derived from castration-resistant metastases. Testosterone levels within metastases from anorchid men [0.74 ng/g; 95% confidence interval (95% CI), 0.59–0.89] were significantly higher than levels within primary prostate cancers from untreated eugonadal men (0.23 ng/g; 95% CI, 0.03–0.44; $P < 0.0001$). Compared with primary prostate tumors, castration-resistant metastases displayed alterations in genes encoding steroidogenic enzymes, including up-regulated expression of FASN, CYP17A1, HSD3B1, HSD17B3, CYP19A1, and UGT2B17 and down-regulated expression of SRD5A2 ($P < 0.001$ for all). Prostate cancer xenografts derived from castration-resistant tumors maintained similar intratumoral androgen levels when passaged in castrate compared with eugonadal animals. Metastatic prostate cancers from anorchid men express transcripts encoding androgen-synthesizing enzymes and maintain intratumoral androgens at concentrations capable of activating AR target genes and maintaining tumor cell survival. We conclude that intracrine steroidogenesis may permit tumors to circumvent low levels of circulating androgens. Maximal therapeutic efficacy in the treatment of castration-resistant prostate cancer will require novel agents capable of inhibiting intracrine steroidogenic pathways within the prostate tumor microenvironment. [Cancer Res 2008;68(11):4447–54]

Introduction

Androgens and the androgen receptor (AR) signaling pathway are intimately associated with prostate carcinogenesis, and testosterone suppression remains the most effective therapy for metastatic prostate cancer (1). However, despite initial clinical responses to castrate or anorchid serum androgen levels, progression to castration-resistant disease is nearly universal. Of importance, recurrent tumors frequently reexpress AR target genes, such as prostate-specific antigen (PSA), and nearly 30% of patients with progressive disease respond to additional hormonal manipulations (2). These findings suggest that many recurrent prostate cancers are neither hormone refractory nor androgen independent, but maintain a clinically relevant reliance on the AR signaling axis. The observation that androgen-regulated genes are frequently reexpressed in castration-resistant prostate tumors has prompted a search for processes contributing to AR activation in the anorchid environment. Proposed mechanisms include amplification and overexpression of the AR, AR gene mutations leading to promiscuous ligand interaction, enhanced AR signal transduction through alterations in coactivators/corepressors, and activation of the AR or downstream regulatory molecules by cross-talk with other signaling pathways (3–5). Importantly, many mechanisms proposed to confer a castration-resistant phenotype either still require or are enhanced by the presence of AR ligands.

The most parsimonious explanation for persistent AR signaling in the setting of anorchid serum testosterone concentrations is the continued presence of intracellular androgens at levels adequate to activate wild-type AR. Studies of primary prostate tissues after medical or surgical castration have measured intraprostatic testosterone or the active metabolite dihydrotestosterone (DHT) in quantities sufficient to stimulate AR-mediated gene expression and protein synthesis (6–8). Whereas the source of residual tissue androgens in the setting of anorchid serum testosterone levels has not been established, intracrine androgen production (the local synthesis or conversion of androgens within a tissue) may play a critical role in maintaining tumoral androgen levels (9). In this regard, the increased expression of genes mediating the conversion of adrenal androgens to testosterone has been reported in bone marrow metastases from men with castration-resistant prostate cancer (10).

In this study, we sought to determine if physiologically relevant androgen levels are present in metastatic soft tissue tumor deposits from patients with castration-resistant prostate cancer. To ascertain the potential for intracrine androgen synthesis, we comprehensively evaluated the expression of genes encoding each enzyme in the steroidogenic pathway leading from cholesterol to testosterone, DHT, and their metabolites. We show that metastatic human prostate cancers from anorchid men express transcripts encoding androgen-synthesizing enzymes and sustain intratumoral androgens at concentrations capable of activating AR target genes and maintaining tumor cell survival.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Materials and Methods

Tissue acquisition and immunohistochemical staining. All procedures involving human subjects were approved by the Institutional Review Board of the University of Washington Medical Center, and all subjects signed written informed consent. Under a University of Washington institutional protocol for the use of excess tissue after surgery, eight matched samples of benign and tumor prostate tissue were obtained from eugonadal patients undergoing radical prostatectomy for localized prostate cancer. In addition, benign prostate tissue was obtained from two patients undergoing cystoprostatectomy unrelated to prostate cancer. Patients with metastatic prostate cancer underwent rapid autopsies under the aegis of the University of Washington Prostate Cancer Donor Autopsy Program as previously described (11). Autopsies were performed within 4 to 10 h of death. Samples of all gross metastatic tumor sites were obtained under sterile conditions, and the site and volume of osseous and nonosseous metastases were recorded. Fresh tissue was snap frozen in liquid nitrogen immediately after harvesting and maintained at −80°C. Tissues used in this study were distant or local soft tissue metastases acquired from eight surgically or medically castrated patients (the latter with documented anorchid serum testosterone levels; <50 ng/dL) and included two to four metastatic lymph node, liver, bladder, or lung tumor deposits per patient. For three patients, nonosseous control tissues not involved with prostate cancer (including skin, liver, and muscle) were simultaneously harvested during the autopsy procedure and were used for analyses of androgen levels. An H&E-stained section of each primary and metastatic tumor sample was examined microscopically for the presence of adenocarcinoma, and areas consisting of >85% tumor tissue were grossly dissected for further use. Benign samples were examined to ensure absence of tumor tissue. Samples were subdivided into pieces ranging from 20 to 50 mg of which three from each tumor sample were used for determination of androgen levels and one was used for RNA isolation. Androgen levels were determined in four sets of the matched tumor and benign prostate specimens, two benign prostate specimens, and in all control and metastatic autopsy tissues. Immunohistochemical staining for AR and PSA was carried out as we have previously described (12), using a polyclonal anti-PSA antibody (DAKO, Inc.) and a monoclonal anti-AR antibody (clone F36.4.1, Biogenex). Negative control immunostains, substituting preimmune immunoglobulin of the same species as that in which the antibody was generated, showed no reaction product.

LuCaP human prostate cancer xenografts. The establishment and maintenance of the LuCaP 23 and 35 xenografts from the lymph node metastases of two individuals with castration-resistant prostate cancer was performed as previously described (13, 14). The LuCaP 96 xenograft was generated from a specimen obtained by transurethral resection of the prostate from a patient 1 mo before documentation of castration-resistant prostate cancer. All lines were established as a component of the University of Washington Rapid Autopsy program, and all lines contain wild-type AR, produce serum PSA, and respond to castration with the subsequent development of castration-resistant or androgen-independent growth. All experiments involving animals were performed in accordance with protocols approved by the University of Washington Institutional Animal Care Use Committee. Castration-sensitive and castration-resistant strains of each xenograft were grown, respectively, in three to five intact (noncastrate) and three to five castrate male CB-17 SCID mice (Charles River Laboratories). Prior terminology describes these tumor phenotypes as androgen dependent and androgen independent, but our data indicate that these terms are misnomers and thus we use the castration-sensitive and castration-resistant nomenclature. When tumors reached ~500 mg in size, the animals were euthanized according to institutional protocol and the xenografts were harvested and flash frozen for determination of tissue androgens and extraction of total RNA. Samples of normal kidney and liver were simultaneously obtained from each intact and castrate animal for determination of tissue androgen levels in nontumor tissue. In addition, a set of LuCaP 35 xenografts were harvested from five mice in which castration-sensitive tumors had been subjected to castration-induced regression with subsequent androgen-independent or castration-resistant regrowth (CS→CR).

Steroid measurements. Androgen levels were determined by mass spectrometry (MS) using methods we have recently described (15). In brief, frozen tissue samples were individually thawed, weighed, and homogenized in PBS. The homogenates were extracted with 8 mL of diethyl ether, and the organic phase was decanted after freezing the aqueous phase in a dry ice/ethanol bath. The organic phase was dried and concentrated with 2 × 0.5 mL ether washes under a stream of purified air. Each individual concentrated extract was dissolved in 1.0 mL redistilled ethanol and stored at −20°C until MS analysis. Samples were added to internal standards: 50 pg of deuterated (D3)-DHT and D3-testosterone, vortexed briefly, and evaporated to dryness. The residue was then reconstituted in 0.5 mL of water before extraction with methylene chloride. The organic phase was removed under nitrogen, and the sample was dissolved in 0.1 mol/L hydroxylamine hydrochloride in 50% methanol/water, vortexed, and heated at 60° for 1 h. Standards for DHT and testosterone were prepared in parallel. The resulting oximes were analyzed by LC-MS-MS using a Waters Aquity HPLC and Premier XE mass spectrometer. Ions monitored were 350>309 and 347>306 for DHT-IS and DHT, respectively, and 307>124 and 304>124 for testosterone-IS and testosterone, respectively. This procedure resulted in a lower limit of quantitation of 100 and 500 attomol on column for testosterone and DHT, respectively. Intraassay coefficients of variation generated using human serum for high-range, mid-range, and low-range samples were 3.5%, 3.1%, and 3.8% for testosterone and 6.3%, 4.3%, and 15.8% for DHT, respectively.

RNA isolation and quantitative reverse transcription–PCR. Samples were individually homogenized in Trizol (Invitrogen), and total RNA was isolated using the RNeasy kit (Qiagen, Inc.), followed by treatment with DNase using the Qiagen RNase-Free DNase Set (Qiagen, Inc.). RNA was quantitated in a Gene-Spec III spectrophotometer (Hitachi), and RNA integrity was evaluated using gel electrophoresis. cDNA was generated from each sample using 2 to 5 μg of total RNA in an oligo dT–primed reverse transcription reaction. Quantitative reverse transcription–PCR (qRT-PCR) reactions were performed in triplicate using an Applied Biosystems 7700 sequence detector with ~5 ng of cDNA, 1 μmol/L of each primer pair, and SYBR Green PCR master mix (Applied Biosystems). Primers specific for genes of interest were designed using the Web-based primer design service Primer3[6] provided by the Whitehead Institute for Biomedical Research, except for *AKR1C1, AKR1C2, AKR1C3* (16), and *17BHSD10* (17) for which previously published primer sequences were used. Sequences are provided in Supplementary Table S1.

Statistical analyses. To account for having multiple samples (i.e., two to four metastatic deposits) with replicate measurements from the same patient, statistical comparison of androgen levels in the human prostate and metastatic autopsy samples was performed using the following linear mixed effects model: $[y_{ij} = \beta_0 + b_i + \beta_1 x_{ij} + \varepsilon_{ij}]$, where y_{ij} is the androgen level (testosterone, DHT), b_i is a random intercept with distribution $N(0, \sigma_b^2)$, x_{ij} indicates tissue type, and ε_{ij} is an individual-specific error term with distribution $N(0, \sigma^2\varepsilon)$; here, i indexes patients and j indexes patient-specific observations. Furthermore, we assume that b_i and ε_{ij} are independent. This model accounts for within-individual correlations, which are assumed to be the same for each individual, and was used to derive P values for the comparison of mean tissue androgen levels among sample types. For each of the three xenograft lines, differences in androgen levels between the castration-sensitive and castration-resistant tumors were assessed by unpaired two sample t tests. P values of <0.05 were considered significant.

For analysis of the qRT-PCR data, the mean cycle threshold (Ct) obtained for each gene was normalized to the expression of the housekeeping gene *RPL13A* in the same sample (the δCt). Reactions with Cts of >35 were considered undetectable for that transcript, and the specificity of amplification in each reaction was assessed based on the melting point of the dissociation curve. Unpaired two-sample t tests were used to

[6] www.cgihttp://jura.wi.mit.edu/rozen/papers/rozen-and-skaletsky-2000-primer3.pdf

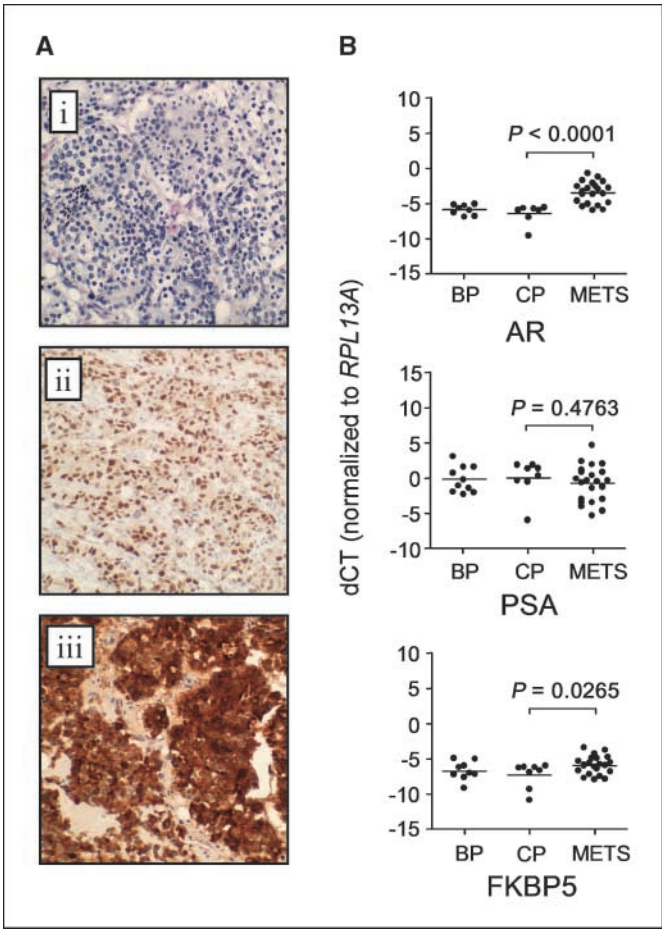

Figure 1. Expression of AR and PSA in castration-resistant metastases. *A,* immunohistochemical analysis of AR and PSA expression in metastatic lymph node foci of prostate adenocarcinoma. Protein expression is reflected as brown chromogen reactivity. *i,* H&E staining demonstrating characteristics of adenocarcinoma; *ii,* AR staining of the same metastasis as in *i* with abundant nuclear AR expression; *iii,* PSA staining of the same metastasis as in *i* with abundant cytoplasmic PSA expression (all images at 10× magnification). *B,* transcript levels for *AR, PSA*, and *FKBP5* in the benign prostate (*BP*), cancer prostate (*CP*), and castration-resistant metastatic tumor (*METS*) samples. Ct for each gene were normalized to the housekeeping gene *RPL13A* in the same sample. The *y* axis is the *RPL13A*-normalized Ct; more positive numbers reflect higher transcript abundance. Unpaired two-sample *t* tests were used to compare the mean Cts for each gene between the cancer prostate and metastatic tumor samples. *P* values of <0.05 were considered significant.

compare the mean δCts for each gene between the primary prostate cancers (n = 8) and metastatic autopsy samples (n = 16–22). Welch's modification of the t test was used if the F test to compare sample variances was significant (but was only applicable to one gene, *UGT2B15*). P values of <0.05 were considered significant. The fold change was calculated by unlogging the difference in mean δCts between the sample groups. Similarities among the human prostate and metastatic autopsy samples based on expression of steroidogenic gene transcripts were assessed by unsupervised, hierarchical, average linkage clustering using Cluster 3.0 software[7] and plotted using TreeView version 1.6.[8] This program organizes genes and samples into a tree structure based on their similarity, in which items are joined by short branches if they are similar to each other and by increasingly longer branches as their similarity decreases. In average linkage clustering, the distance between two items x and y is the mean of all pairwise distances between items contained in x and y and therefore provides a visual estimate of the similarity among different items in a sample.

Results

Expression of the androgen-AR signaling axis in castration-resistant prostate cancer metastases. To study mechanisms responsible for prostate cancer progression in the setting of anorchid serum testosterone levels, we first sought to evaluate the integrity of the AR signaling axis within tumor metastases by examining the expression of *AR* and the androgen-regulated genes *PSA* and *FKBP5*. Using a rapid postmortem tissue collection protocol (11), we obtained metastatic tumor samples from patients

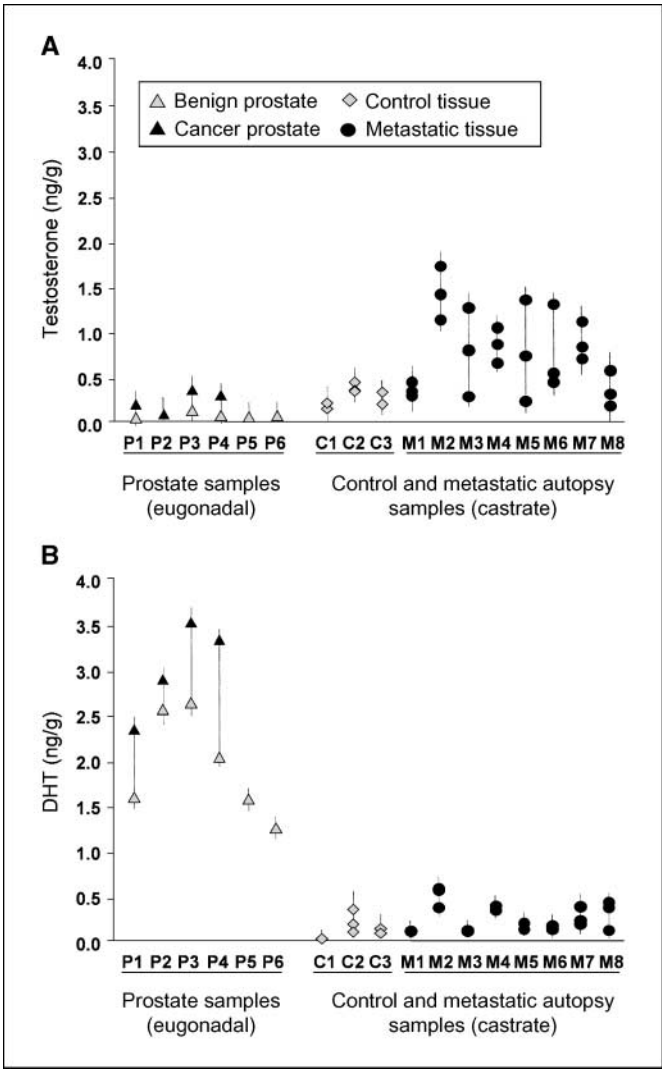

Figure 2. Quantitation of tissue androgens in primary and castration-resistant metastatic prostate tumors. Testosterone (*A*) and DHT (*B*) levels were evaluated by MS in paired benign and cancer prostate tissues from four eugonadal patients undergoing prostatectomy (*P1–P4*), in benign prostate tissue from two patients undergoing cystoprostatectomy for bladder cancer (*P5* and *P6*), and in multiple metastatic tumor deposits obtained at autopsy from each of eight patients with castration-resistant prostate cancer (*M1–M8*). Control tissues not involved by tumor were simultaneously obtained from a subset of patients during the autopsy procedure (*C1–C3*). Each tissue sample was subdivided into triplicate samples that were separately processed. *Points,* mean of each triplicate.

[7] http://bonsai.ims.u-tokyo.ac.jp/-mdehoon/software/cluster/software.htm
[8] http://rana.lbl.gov/EisenSoftware.htm

with progressive disease despite either surgical castration or medical castration with clinically documented anorchid serum testosterone concentrations (≤50 ng/dL). Castration-resistant tumors generally showed intense nuclear staining for AR, as well as strong cytoplasmic PSA reactivity (Fig. 1*A*), although heterogeneity in expression was found in a subset of tumors as we and others have previously described (11, 18). Quantification of *AR, PSA*, and *FKBP5* gene expression by qRT-PCR showed increased expression of *AR* and equivalent levels of *PSA* and *FKBP5* in the castration-resistant metastases compared with benign prostate tissue and primary prostate cancers (Fig. 1*B*). These data are consistent with prior reports (19–22) and show the continued activity of the AR signaling axis in most castration-resistant tumors despite anorchid serum androgen levels.

Testosterone levels in castration-resistant metastases and primary prostate tumors. To investigate whether the AR signaling activity observed in the castration-resistant prostate cancer metastases could be due to the presence of intratumoral androgens, we obtained multiple soft tissue metastatic deposits from patients with progressive disease and anorchid serum testosterone concentrations (using the rapid postmortem tissue collection protocol described above; ref. 11). Benign samples (control tissues not involved with tumor, including skin, muscle, and liver samples) were simultaneously obtained from a subset of patients. We also evaluated paired cancer and benign prostate tissues from untreated eugonadal patients undergoing prostatectomy and prostate tissue from patients without prostate cancer undergoing cystoprostatectomy for bladder cancer. Multiple samples from each tissue were separately processed, and androgen concentrations were quantified by MS.

Testosterone concentrations in metastatic prostate tumors from anorchid patients ranged from 0.2 to 1.78 ng/g (Fig. 2*A*) up to 4-fold higher than levels in noncancerous control tissues acquired at autopsy or in primary prostate cancer samples from eugonadal patients (Table 1; $P < 0.0001$). Higher levels of testosterone and DHT were observed in primary prostate cancers compared with paired benign prostate tissues ($P = 0.01$ for testosterone and $P < 0.0001$ for DHT; Fig. 2*A* and *B*; Table 1). Primary prostate tumors from eugonadal patients retained the 10:1 to 20:1 ratio of DHT to testosterone observed in benign prostate tissues. In contrast, this ratio was markedly reversed in metastatic tumors, which displayed a DHT/testosterone ratio of ~0.25:1, consistent with a prior report evaluating locally recurrent, castration-resistant primary prostate tumors (7). Importantly, the testosterone concentrations measured in the prostate cancer metastases are higher than those in the nonprostatic control tissues, exceed mass equivalent concentrations in the serum of these anorchid men, and are well within a range known to stimulate the AR and support prostate cancer cell proliferation (22–24).

Table 1. Mean tissue androgen levels in castration-resistant metastases from anorchid patients versus primary prostate tissues from eugonadal men

Tissue source	Testosterone, ng/g (95% CI)*	DHT, ng/g (95% CI)*
Benign prostate	0.04 (0.00–0.24)	1.92 (1.63–2.21)
Cancer prostate	0.23 (0.03–0.44)	2.75 (2.45–3.04)
Control tissue †	0.10 (0.00–0.26)	0.05 (0.00–0.30)
Metastatic tissue	0.74 (0.59–0.89)	0.25 (0.00–0.50)

Abbreviation: 95% CI, 95% confidence interval.

*$P < 0.0001$ for comparison among benign prostrate versus cancer prostrate, control tissue versus metastatic tissue, and cancer prostrate versus metastatic tissue for both testosterone and DHT (except $P = 0.01$ for difference in testosterone between benign prostrate and cancer prostrate). Mean values, confidence intervals, and P values were calculated using a linear mixed-effects model to account for multiple observations and intraindividual correlations.

† Nontumor tissues obtained concurrently with tumor metastases from men with castration-resistant prostate cancer.

Alterations in transcripts encoding steroidogenic enzymes in castration-resistant metastases. To determine whether prostate cancer metastases may be capable of synthesizing androgens *de novo*, we quantified transcripts encoding each enzyme involved in the sequential biosynthesis of testosterone and DHT from cholesterol precursors (Fig. 3*A*). Compared with untreated primary prostate tumors, castration-resistant metastases showed significant increases in the expression of *FASN, HSD3B1, HSD3B2, CYP17A1, AKR1C3*, and *HSD17B3*, key enzymes required for metabolism of progestins to adrenal androgens and their subsequent conversion to testosterone. Representative results are shown in Fig. 3*B*, and the data for all genes are summarized in Table 2. Consistent with the marked reversal of the DHT/testosterone ratio in the metastatic samples, they expressed significantly lower levels of *SRD5A2*, which catalyses the conversion of testosterone to DHT, and higher levels of *UGT2B15* and *UGT2B17*, which mediate the irreversible glucuronidation of DHT metabolites (Fig. 3*B*; Table 2). Interestingly, marked up-regulation of *CYP19A1*, which mediates the aromatization of testosterone to estradiol, was also observed in the metastases and is consistent with prior reports demonstrating up-regulated expression of aromatase in malignant versus benign prostate epithelium (25).

Hierarchical clustering of tumors based on expression of steroidogenic enzyme transcripts clearly distinguished primary prostate cancers and benign prostate tissue from castration-resistant metastases, with metastatic samples generally clustering by patient of origin (Supplementary Fig. S1*A*). This observation suggests that adaptive modulation of steroidogenic pathways to the castrate environment may occur within the tumor before initiation of the metastatic cascade. Importantly, transcripts encoding the full complement of enzymes comprising the steroidogenic pathway were detectable in the majority of primary and metastatic prostate tumors examined (Supplementary Fig. S4*B*).

Intratumoral androgen levels in prostate cancer xenografts grown in intact and castrate mice. We next evaluated androgen levels in a series of prostate cancer xenografts grown in noncastrate (intact) and castrate male SCID mice. Xenografts were derived from castration-resistant lymph node metastases (LuCap23.1 and LuCap35) or primary prostate tumor (LuCap96). Castration-sensitive xenografts were passaged in intact mice, whereas isogenic castration-resistant variants were maintained in castrate hosts. Remarkably, testosterone levels in the LuCap23.1 and LuCap35 xenografts derived from lymph node metastases were equivalent whether tumors were grown in castrate or intact mice (Fig. 4; Supplementary Table S2). Testosterone levels in the prostate-derived LuCap96 were higher in tumors from intact eugonadal mice, but remained easily detectable in the castration-resistant tumors from the castrate hosts. Similarly, while more

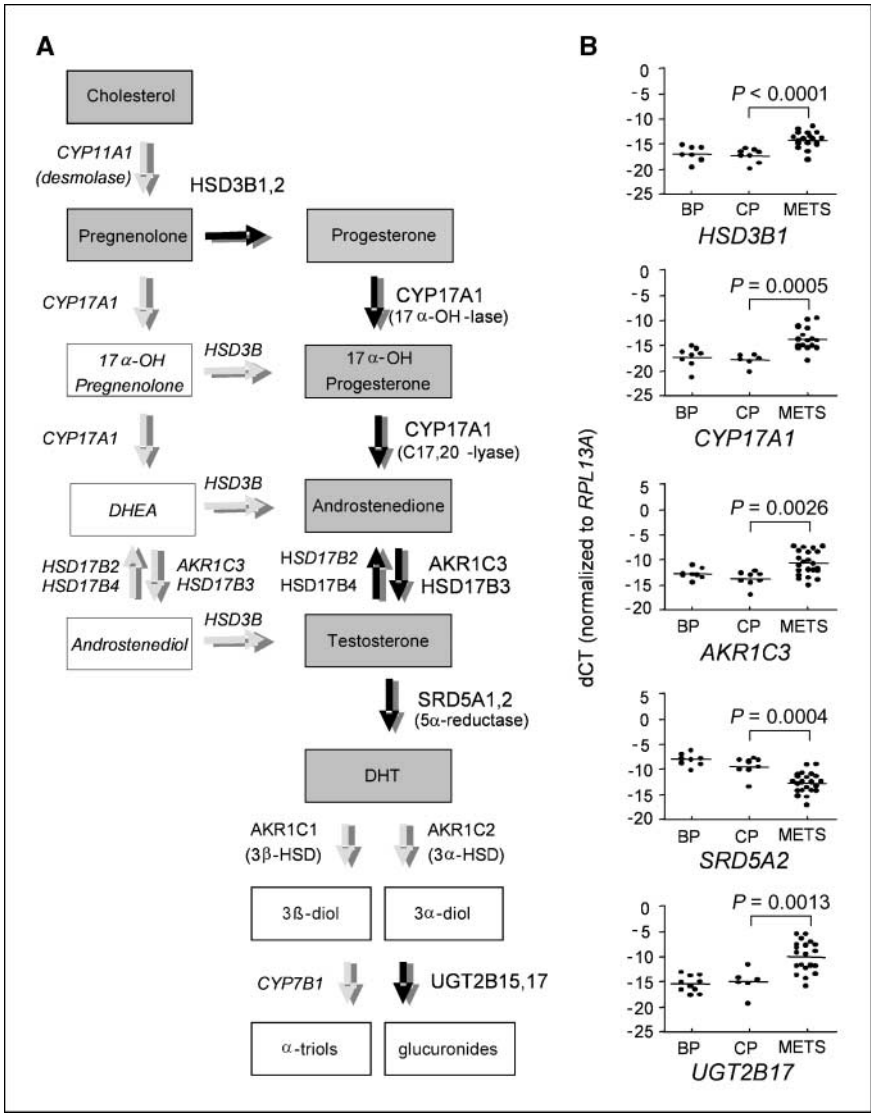

Figure 3. Expression of steroidogenic enzyme transcripts in primary and metastatic prostate tumors. *A,* the enzymatic pathways mediating the sequential biosynthesis and metabolism of testosterone and DHT from cholesterol and progestin precursors were evaluated by qRT-PCR. Bold arrows denote key metabolic steps (*colored enzymes*) for which transcript levels were significantly altered in the castration-resistant prostate cancer metastases versus primary prostate tumors. *B,* representative dot plots for the key metabolic enzymes highlighted in *A* (*HSD3B1, CYP17A1, AKR1C3, SRD5A2*, and *UGT2B17*). Transcript levels for the indicated enzymes were evaluated in the benign prostate, cancer prostate, and metastatic tumor samples; Cts for each gene were normalized to expression of the housekeeping gene *RPL13A* in the same sample. The *y* axis is the *RPL13A*-normalized Ct, where more positive numbers reflect higher transcript abundance. Unpaired two-sample *t* tests were used to compare the mean Cts for each gene between the cancer prostate and metastatic tumor samples. *P* values of <0.05 were considered significant.

heterogeneous among replicates, DHT levels in the majority of castration-resistant xenograft samples from castrate mice were equivalent or higher than those measured in the isogenic castration-sensitive xenografts grown in intact mice. Testosterone and DHT levels in normal tissue samples (kidney and muscle) from either the intact or castrate host animals did not approach those in tumor tissue.

Discussion

The mechanisms by which advanced prostate cancers maintain AR-mediated gene expression after castration are poorly defined. In this study, we determined that soft tissue metastases from castration-resistant prostate cancers exhibit elevated testosterone concentrations compared with untreated primary tumors. The processes responsible for sustaining intratumoral androgen levels in the setting of systemic testosterone suppression have yet to be determined. The potential contribution of adrenal androgens to prostate tumor growth is well-recognized, and inhibiting this androgen source is a major treatment focus for castration-resistant tumors. Drugs which were serendipitously found to target steroid synthesizing enzymes in the adrenal gland have shown significant, albeit short-term responses (26, 27). Adrenalectomy and hypophysectomy also have efficacy in a limited number of patients (28). Besides the uptake and conversion of circulating adrenal androgens, prostate cancer metastases may also be capable of *de novo* androgen biosynthesis from cholesterol and/or progesterone precursors (19).

Our data show that transcripts encoding the full complement of enzymes involved in the sequential biosynthesis of testosterone, and DHT from cholesterol precursors were expressed in the majority of castration-resistant metastatic tumors examined. Furthermore, the specific steroidogenic genes altered in our data confirm and extend previous studies of gene expression in castration-resistant bone marrow metastases (10) to include up-regulated expression of *CYP17A1*, a critical enzyme mediating sequential steps in the production of adrenal androgens from progestins, as well as increased expression of *HSD17B3*, which mediates the same metabolic step as *AKR1C3*, the conversion of androstenedione to testosterone (29). A clear limitation of our study is the degree to which transcript alterations correlate with changes in biosynthetic enzyme activity. Whereas demonstration

Table 2. Relative expression of steroidogenic enzymes in castration-resistant metastases versus primary prostate tumors

Gene	Fold change*	*P*
STAR	5.1	0.0105
FASN	9.6	0.0003
CYP11A	−1.1	0.8362
CYP17A	16.9	0.0005
3BHSD1	8.5	<0.0001
3BHSD2	7.5	0.0091
17BSHD2	8.2	0.0137
17BHSD3	8.7	<0.0001
17BHSD4	4.8	0.0019
AKR1C1	2.7	0.0601
AKR1C2	1.1	0.7895
AKR1C3 †	8.0	0.0026
SRD5A1	2.63	0.0050
SRD5A2	−9.4	0.0005
CYP19A1	30.3	<0.0001
UGT2B15	10.0	0.0779
UGT2B17	34.7	0.0013

*Fold change calculated by unlogging the difference in mean cycle threshold between the sample groups. *P* values derived from unpaired two-sample *t* tests.
† Also termed *17BHSD5*.

of enzymatic function is beyond the scope of the present work, the presence of transcripts encoding each gene in the androgen biosynthetic pathway is a necessary prerequisite to steroidogenesis, and a comprehensive assessment of genes in this pathway has not been previously reported in prostate cancer metastases.

The increased expression of AR transcripts in the castration-resistant metastases may be of particular importance in promoting tumor cell growth at the androgen levels detected in these samples. Gregory et al. have shown that prostate cancer cell lines derived from recurrent tumors show increased expression and stability of the AR, in association with an increased sensitivity to proliferation at low levels of DHT. Furthermore, Chen et al. have shown that increased AR expression is instrumental in the progression from androgen-dependent to castration-resistant growth in a xenograft model, with the degree of AR up-regulation observed sufficient to allow tumor cell proliferation in 80% lower androgen concentrations (3). Importantly, ligand binding was required for hormone refractory growth, and modest increases in AR expression were sufficient to support signaling in a low androgen environment. Conversely, AR protein expression was equivalent in a series of locally recurrent prostate tumors compared with benign prostate epithelium (7). As such, increased AR message may not necessarily result in uniformly higher AR protein expression, but may contribute to maintaining AR protein expression at levels required for sustaining tumor cell growth.

Our study found a marked reversal in the ratio of testosterone to DHT in the castration-resistant tumor metastases compared with the primary prostate tissues. This observation is consistent with the study of Mohler et al., in which testosterone levels in locally recurrent, castration-resistant primary prostate tumors were maintained at levels found in untreated benign prostatic hyperplasia tissues whereas DHT levels were significantly decreased (7). Although DHT is ~10-fold more potent than testosterone in binding and activating AR (30), kinetic experiments have shown that testosterone at high concentrations interacts with AR similarly to DHT (31). In studies evaluating androgen-induced prostate regrowth in castrated rats, Wright et al. found that 1.6-fold to 1.9-fold increases in testosterone compared with DHT were sufficient to achieve comparable measures of prostate regrowth (32). Conversely, Xu et al. have shown that dutasteride treatment inhibited the growth of Dunning R-3327H rat prostate tumors and

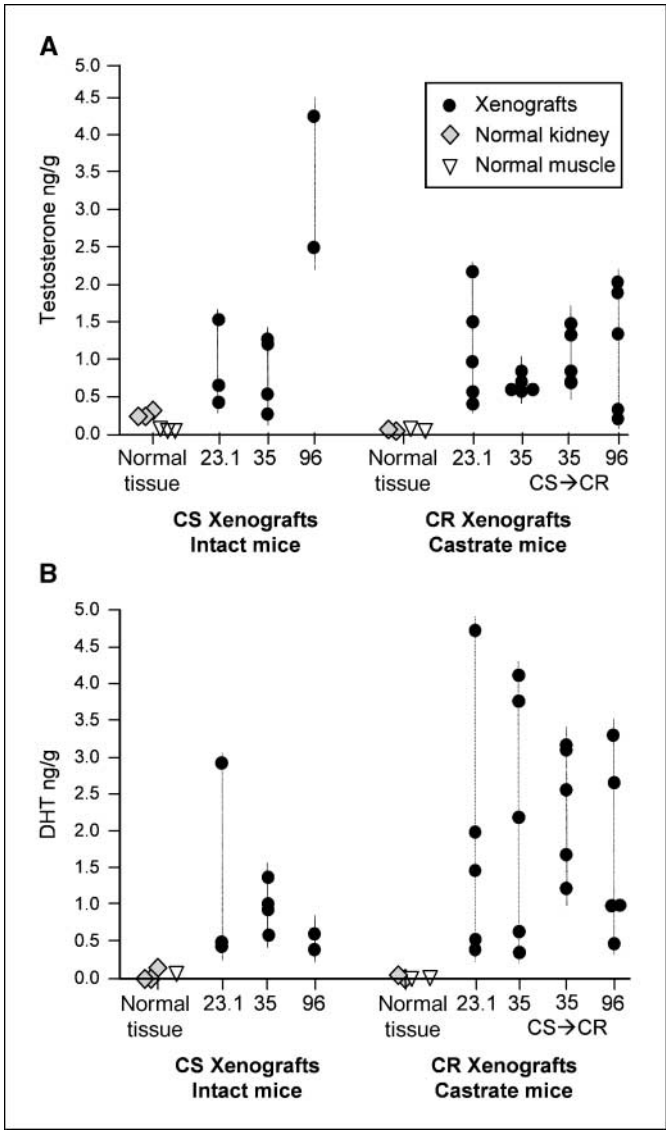

Figure 4. Androgen levels in LuCaP prostate cancer xenografts grown in castrate and intact mice. Testosterone (*A*) and DHT (*B*) levels were measured by MS in castration-sensitive (*CS*) and castration-resistant (*CR*) variants of the indicated xenografts. Two to five castration-sensitive and castration-resistant tumors of each line were passaged in noncastrate (intact) or castrate male SCID mice, respectively, as indicated. The LuCaP35 CS→CR samples are xenografts initially passaged as castration-sensitive in intact mice, which then responded to castration with castration-resistant growth. *Points,* mean value for an individual xenograft harvested from one mouse. Samples were subdivided and assayed in duplicate. Androgen levels were also evaluated in normal kidney and muscle tissues simultaneously obtained from each set of castrate and intact animals. Mean androgen levels for each castration-sensitive and castration-resistant xenografts are presented in Supplementary Table S2.

LNCaP human prostate xenografts, despite a concomitant 2-fold increase in tissue testosterone levels. However, whereas dutasteride reduced tumor DHT levels to essentially the same nadir as castration, it did not produce the same magnitude of tumor growth inhibition as castration. Moreover, the combination of dutasteride plus castration produced greater growth inhibition of LNCaP xenografts than either castration or dutasteride alone, suggesting that whereas testosterone may not be as potent as DHT, it is capable of stimulating prostate tumor growth. In earlier studies evaluating the growth response of androgen-dependent xenografts, Van Weerden et al. found that androgen-induced increases in tumor cell growth only occurred when tissue androgen levels exceeded a critical threshold value, corresponding to ~0.9 ng/g of DHT (33). Although higher than the DHT levels observed in the tumor metastases in our study, this value may reflect the androgen dependence of the xenografts evaluated by van Weerden and does not preclude the possibility of tumor cell stimulation at the lower androgen levels detected in the castration-resistant metastases.

The detection of testosterone as the primary androgen in the castration-resistant metastases may reflect the subtotal decrease in tumoral SRD5A activity that has been consistently observed in prostate tumors since its original demonstration in lymph node metastases and primary prostate cancers by Klein et al. (34) More recently, studies have shown a decrease in SRD5A2 expression in neoplastic compared with benign prostate tissues, accompanied by a relative shift in expression and enzymatic activity to SRD5A1 in primary and recurrent prostate tumors (10, 35–39). These findings are consistent with our measurements of ~9-fold decrease and ~2.5-fold increase in *SRD5A2* and *SRD5A1* expression, respectively, in the tumor metastases. Alternatively, the concentrations of DHT detected in the tumor metastases we analyzed may actually be an underestimate of the *in vivo* tumor androgen levels, as several studies have shown that DHT levels in autopsy samples may be factitiously low due to ongoing postmortem androgen metabolism or degradation (40, 41). We cannot exclude this possibility, which may also underlie the relatively higher levels of DHT observed in the prostate cancer xenografts, as these tissues can be immediately frozen upon harvesting. Interestingly, the androgen levels detected in the castration-resistant xenografts suggests these tissues may be capable of *de novo* androgen biosynthesis, as some (albeit not all) studies have suggested that castrate rodents do not make adrenal androgens due to a lack of *CYP17* expression in the rodent adrenal gland (42, 43).

In conclusion, we propose that metastatic prostate cancers may adapt to low systemic testosterone levels by maintaining intratumoral androgens through the modulation of enzymes involved in intracrine steroidogenesis and androgen catabolism. Our data suggest that secondary hormonal manipulations and pharmacologic inhibitors of androgen biosynthesis derive a component of their activity by directly targeting intratumoral androgen production. This mechanism may explain the relatively high response rates observed in recent clinical studies evaluating specific CYP17A1 inhibitors for castration-resistant prostate cancer when compared with historical rates observed with adrenalectomy (28, 44–46). Moreover, elevated tumoral androgen levels may underlie the lack of substantial survival benefit associated with the use of AR antagonists, as agents such as bicalutamide have an affinity for the AR which is 30-fold lower than the endogenous ligand (47, 48). These observations strongly suggest that improving clinical outcomes in castration-resistant prostate cancer will require combinatorial treatment strategies designed to abrogate intracrine and systemic contributions to the tumoral androgen axis. Furthermore, the application of agents targeting intratumoral androgen production during the treatment of androgen-sensitive prostate cancer may delay or prevent the progression to castration-resistant disease.

Disclosure of Potential Conflicts of Interest

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